AWARD NUMBER: W81XWH-11-1-0068

TITLE: Synthesis and Evaluation of Novel RSK Inhibitors in a Living Human Breast Model

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REPORT DATE: Sept 2015

TYPE OF REPORT: Final Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2015	Final Summary	01 Jul 2011 – 30 Jun 2015

4. TITLE AND SUBTITLE Synthesis and Evaluation of Novel RSK Inhibitors in a Living Human Breast Model

5a. CONTRACT NUMBER W81XWH-11-1-0068

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

RSK (p90 Ribosomal S6 kinase) is critical for breast cancer proliferation and thus a promising target for therapeutic intervention. A highly specific inhibitor of RSK, called SL0101, was previously discovered but found to possess poor biological stability and potency. The purpose of this project is to identify a drug for breast cancer based on SL0101 that works by inhibiting RSK, by designing and chemically synthesizing analogues of SL0101 that improve on its biological stability and potency and ultimately evaluating them in a living human breast tissue model for anticancer activity. The major findings have been the discovery of analogues of Sl0101 that are more potent and more biologically stable thab Sl0101 and the solution of and X-Ray crystal structure of SL0101 in complex with RSK2. These discoveries represent significant steps toward the goal of identifying an analogue of SL0101 that could be used as a drug for breast cancer.

15. SUBJECT TERMS
breast cancer, p90 Ribosomal S6 kinase, TNBC, SL0101, p90RSK, RSK

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	18	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

The p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases, comprising at least four isoforms (RSKs 1-4), has been shown to be critical for breast cancer cell proliferation (*1*–*3*). In 2005 Dr. Lannigan reported the first specific inhibitor of RSK, SL0101 (**1**, Figure 1) (*3*). SL0101 inhibits RSK in both the breast cancer cell line MCF7 and the normal breast cell line MCF-10A, but only inhibits the proliferation of the breast cancer cell line (*1*–*3*). This indicates that breast cancer cells have become dependent on RSK and thus identifies RSK as a potential new target for cancer therapeutics. SL0101, given its exquisite specificity for RSK, is an attractive lead compound for medicinal chemistry efforts aimed at discovering a breast cancer drug that acts by inhibiting RSK. However, SL0101 itself is not suitable for further development as a drug for two reasons. First, we have determined that SL0101 has a very short biological half-life in mice (0.4 h at 2.5 mg/kg IP). In order to develop a drug this half-life must be improved so that the drug persists in the patient long enough to act on RSK. Second, SL0101 is a potent RSK inhibitor (IC_{50} ~ 0.5 μM), but a much less potent inhibitor of the proliferation of MCF7 breast cancer cells (EC_{50} = 50 μM), suggesting that it does not readily pass through the cell membrane (*3*). In order to develop a drug, the potency against MCF7 cells must be improved. The scope of this project is to design and chemically synthesize analogues of SL0101, with the assistance of a computational model of SL0101 bound to RSK, that improve on these deficiencies and to evaluate them both in vitro and in vivo with the goal of identifying a new breast cancer drug that acts by inhibiting RSK. If warranted, the best analogues will be evaluated in our new living human breast tissue model (*4*) to gain insights in to the role of RSK in breast cancer that might not be gleaned from in vitro and cell-based assays.

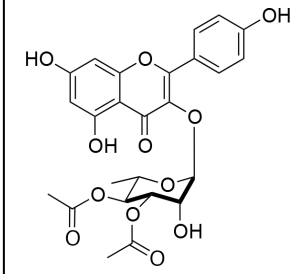


Fig 1. SL0101, a highly specific inhibitor of p90 ribosomal S6 kinase (RSK)

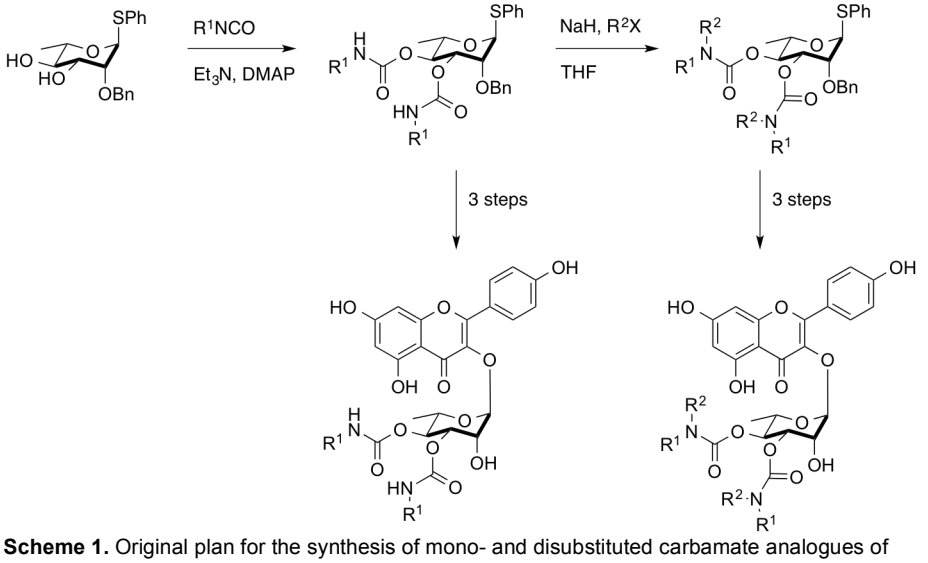
2. Keywords

Breast cancer, TNBC, kinase, SL0101, RSK, p90RSK, small molecule, MCF-7, MCF-10A

3. Overall Project Summary

Task 1: Synthesis and biological evaluation of 3',4'-carbamate analogues of SL0101. I will first synthesize N-methyl and N,N-dimethyl carbamates or related analogues. I will then evaluate them in in vitro and cell-based assays. If their biological activity is sufficient to warrant further analogues in this series, I will then synthesize additional N-alkylated analogues. (Timeframe: months 1-12)

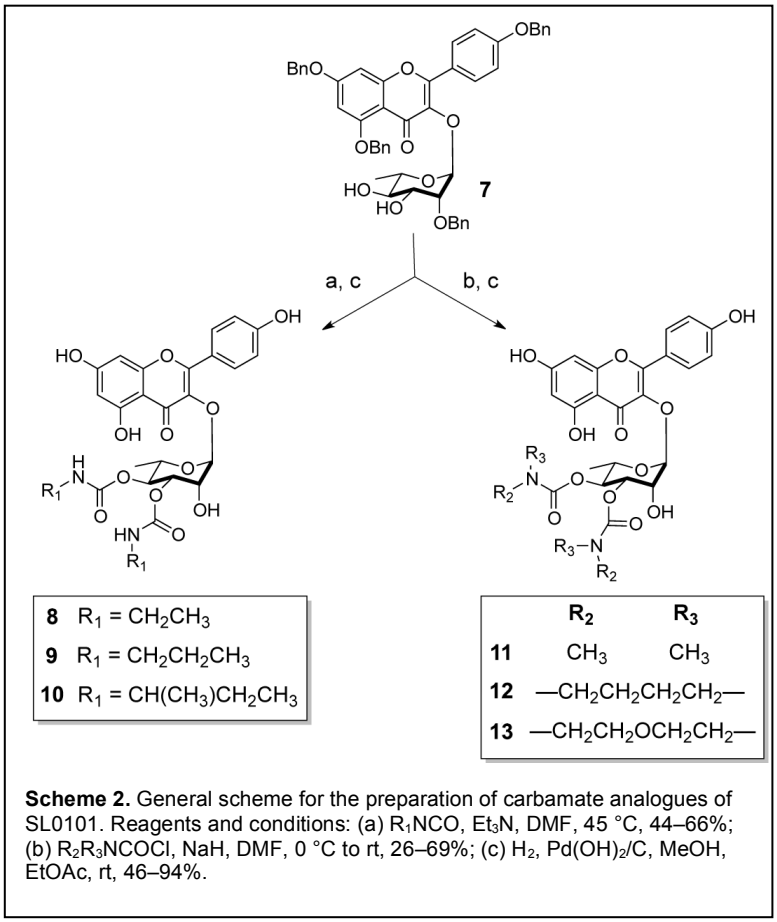
This task was completed within the allotted timeframe, and the results were published in early 2012 (*5*). Details for each subtask follow and the manuscript and experimental details are attached in the appendix. The purpose of the task was to test the hypothesis that the acetates of the sugar portion of SL0101, which would likely be prone to hydrolysis in vivo, could be replaced by less labile carbamates, which would render an analogue more biologically stable than SL0101 while retaining the parent compound's ability to specifically inhibit RSK.



Scheme 1. Original plan for the synthesis of mono- and disubstituted carbamate analogues of SL0101

1a. Synthesis of 3',4'-N-methylcarbamate analogue, 3',4'-N,N-dimethylcarbamate analogue, or related analogues of SL0101. (months 1-6)

Originally, the plan for the chemical synthesis of monosubstituted carbamate analogues such as the proposed 3',4'-*N*-methyl carbamate analogue and *N*,*N*-disubstituted carbamate analogues was to proceed according to the synthetic route outlined in Scheme 1. This plan called for the installation of the carbamate substitution at an early stage in the synthesis of the analogues, starting from known intermediate **2** (*6*) which would require three news synthetic steps to be performed in order to synthesize each new analogue. Rather than follow this proposed synthetic route, I adopted a revised plan that would allow me to synthesize the desired analogues in a much shorter time frame. This revised synthetic route as it applies to monosubstituted carbamates is shown in Scheme 2. Starting from the more advanced synthetic intermediate **7** (*7*), monosubstituted carbamates could be installed by reacting the appropriate isocyanate with the diol **7** in the presence of triethylamine to provide carbamates **8**-**10**. Hydrogenolysis of the benzyl protecting groups gave the completed analogues in one additional synthetic step. This modified route, requiring only two new synthetic steps per analogue, allowed the completion of three new monosubstituted carbamate analogues in the time originally allotted for synthesizing just one. The new analogues are the ethyl carbamate analogue **8**, the n-propyl carbamate analogue **9**, and the sec-butyl carbamate analogue **10**.



Scheme 2. General scheme for the preparation of carbamate analogues of SL0101. Reagents and conditions: (a) R_1NCO, Et_3N, DMF, 45 °C, 44–66%; (b) R_2R_3NCOCl, NaH, DMF, 0 °C to rt, 26–69%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, rt, 46–94%.

The modified synthetic route could also be applied to the synthesis of *N*,*N*-disubstituted carbamate analogues of SL0101 in fewer steps than the originally proposed route. Deprotonation of diol **7** with sodium hydride followed by treatment of the resulting bis-alkoxide with the appropriate dialkylcarbamoyl chloride gave *N*,*N*-disubstituted carbamate analogues **11-13** (Scheme 3). As in the case of the monosubstituted carbamates, the revised synthetic route allowed for the synthesis of three new analogues in the time allotted for just one. The new analogues synthesized are the dimethyl carbamate analogue **11**, the pyrrolidinyl carbamate analogue **12**, and the morpholino carbamate analogue **13**.

1b. In vitro evaluation of analogues. (months 6-9)

The six new carbamate analogues of SL0101 were evaluated for their ability to inhibit RSK2 activity in an in vitro kinase assay (Table 1). The analogues were all either as potent or slightly (2- to 3-fold) less potent that SL0101. That the structure of the acetate replacement did not substantially affect the ability to inhibit RSK2 was not surprising, as this is consistent with what had been seen for previous analogues (*7*).

Compound	RSK2 IC_{50} (μM)	MCF7 IC_{50} (μM)
1	0.583 (0.489 to 0.696)	45.6 (42.7 to 48.8)
8	1.13 (0.876 to 1.46)*	77.0 (71.6 to 82.7)*
9	0.869 (0.649 to 1.16)	46.4 (43.2 to 50.0)
10	1.92 (1.29 to 2.86)*	53.3 (50.6 to 56.2)*
11	0.493 (0.355 to 0.684)	PS
12	0.356 (0.255 to 0.496)	PS
13	1.43 (1.09 to 2.04)*	> 100

Table 1. Potency of analogues in in vitro kinase and cell-based assays. IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; n=3 in triplicate; * p <0.05; PS; partially soluble.

1c. Evaluation of growth inhibition activity of analogues in human cancer MCF7 and normal human MCF-10A cell lines. (months 6-9)

The ability of the six new carbamate analogues to inhibit the growth of the MCF7 cancer cell line was determined (Table 1). The three monosubstituted carbamate analogues were all as potent or slightly less potent than SL0101 in this cell proliferation assay. Two of the disubstituted carbamate analogues were only partially soluble in the MCF7 cell culture media, and were not tested as the insolubility would confound the assay results. The third disubstituted carbamate analogue, **13**, was soluble but was completely unable to inhibit the proliferation of MCF7 cells.

The original hypothesis was that these analogues would be more biologically stable than SL0101. We devised an assay that would allow us to test this hypothesis in vitro. By treating MCF7 cells with SL0101 or each of the new monosubstituted carbamate analogues and monitoring their growth over a period of several days (2-, 4-, and 6-day time points), I could observe whether the inhibition of cell growth was sustained over a long time course. An analogue that was stable in vitro would be expected to still inhibit the growth of cells even at the 6-day time point. As predicted, SL0101 was not biologically stable in vitro, as the cells exposed to it recovered their ability to grow within 4 days (Figure 2). Encouragingly, two of the new monosubstituted carbamate analogues, **9** and **10**, continued to inhibit the growth of the cells even after 6 days, indicating that as predicted they are more biologically stable than SL0101 in vitro.

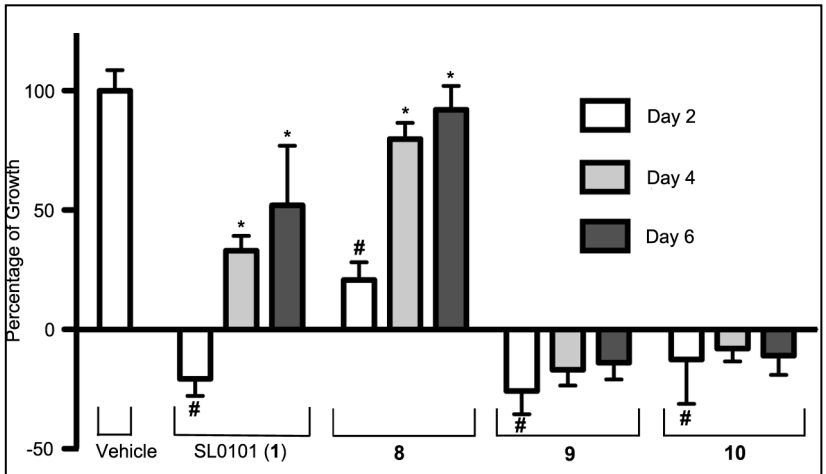


Figure 2. In vitro determination of analogue stability. The inhibitor was added to MCF7 cells when they were plated and percentage of growth was determined for the indicated time points. The inhibitor concentration was 100 μM. Analogues demonstrating improved biological stability continued to inhibit growth on day 6. (n=3 in quadruplicate, # p≤0.05 on day 2 when compared to vehicle on day 2, * p≤0.05 when compared to 2 day treatment with the same analogue.

Finally, the two analogues shown to be biologically stable in vitro, **9** and **10**, were tested for their ability to inhibit the growth of the normal human breast cell line MCF-10A. We have previously shown that the specificity of an analogue for RSK can be evaluated by determining its antiproliferative activity in normal (MCF-10A) vs cancer (MCF7) cell lines (*3*, *8*). Analogues that are specific for RSK inhibit the proliferation of the cancer cell line but not the normal cell line. Like SL0101, at a concentration of 50 μM each analogue was completely unable to inhibit the growth of MCF-10A cells, even though at the same concentration they were each able to inhibit the growth of MCF7 cells (Figure 3). This is the desired result, as it indicates that the new analogues are, like SL0101, highly specific for RSK.

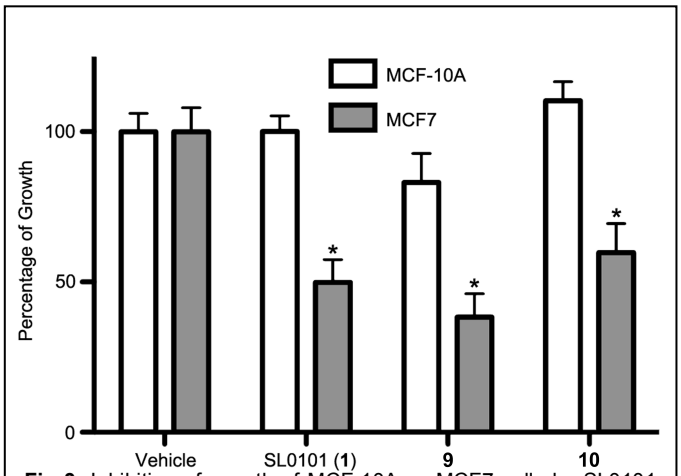


Fig 3. Inhibition of growth of MCF-10A vs MCF7 cells by SL0101 and select analogues. The inhibitor concentration was 50 μM. (n=3 in quadruplicate, * p≤0.05 when compared to vehicle.

The discovery of analogues **9** and **10**, that are more biologically stable than SL0101 but retain specificity for RSK, is a major advance toward the goal of developing a drug for breast cancer that works by inhibiting RSK. These new analogues are potential candidates for in vivo testing.

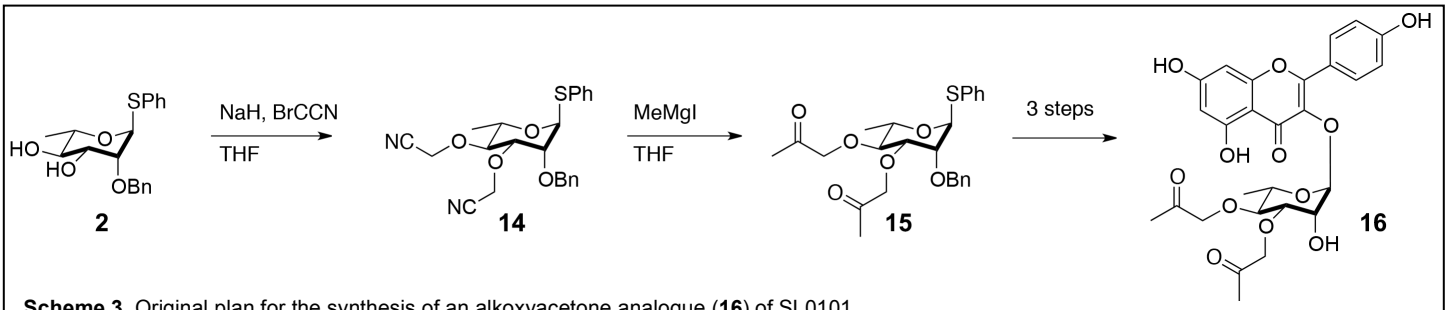
1d. If warranted, synthesis of additional N-alkylated analogues. (months 9-12)

Given that six analogues were synthesized in task 1a rather than the originally planned two, and that two of these analogues achieved the goal of being as potent as SL0101 but more biologically stable in vitro, it was decided that the synthesis of additional *N*-alkylated analogues was not necessary.

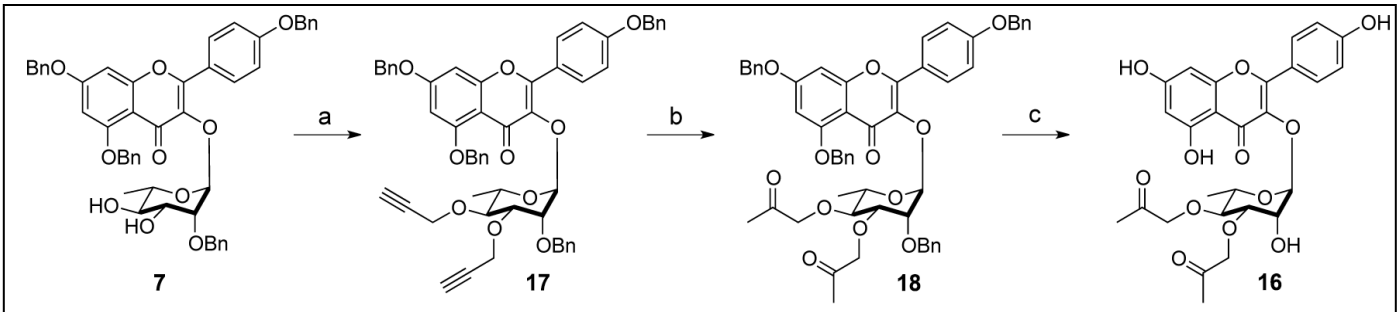
Task 2: Synthesis and biological evaluation of a 3',4'-alkoxypropanone or related analogue of SL0101. (Timeframe: months 1-9)

This task was accomplished within the allotted timeframe, and the results were published in early 2012 (*4*). Details for each subtask follow and the manuscript and experimental details are attached in the appendix. The purpose of the task was to test the hypothesis that analogues of SL0101 in which the 3" and 4" acetates are replaced by alkoxyacetones would be more biologically stable than SL0101 while retaining specificity for RSK.

2a. Synthesis of a 3'4'-alkoxypropanone or related analogue. (months 1-6)



Scheme 3. Original plan for the synthesis of an alkoxyacetone analogue (**16**) of SL0101.



Scheme 4. Synthesis of an alkoxyacetone analogue of SL0101. Reagents and conditions: (a) NaH, propargyl bromide, THF, 0 °C to rt, 66%; (b) $Hg(OAc)_2$, PPTS, water, acetone, rt, 62%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, 50%.

The originally-planned synthetic route to a 3',4'-alkoxypropanone analogue involved alkylation of intermediate **2** with bromoacetonitrile followed by addition of methyl Grignard and subsequent hydrolysis to provide intermediate **15** which could be elaborated by known methods (*6*) to desired analogue **16** (Scheme 3). Ultimately, a different synthetic route was chosen due to a higher predicted likelihood of success (Scheme 4). Diol **7** was first alkylated with propargyl bromide to give bis-alkyne **17**. The terminal alkynes were subjected to mercury-catalyzed hydration to provide the desired 3',4'-alkoxypropanone intermediate **18**, which could be converted to the desired analogue **16** by hydrogenolysis of the benzyl protecting groups.

2b. In vitro evaluation of analogue. (months 6-9)

Analogue **16** was evaluated for its ability to inhibit RSK2 in an in vitro kinase assay. It inhibited RSK2 with an IC_{50} of 0.252 μM (95% CI 0.189 to 0.336 μM), making it two-fold more potent than SL0101.

2c. Evaluation of growth inhibition activity of analogue in human cancer MCF-7 and normal human MCF-10A cell lines. (months 6-9)

Analogue **16** was evaluated for its ability to inhibit the growth of the MCF7 cell line. It inhibited proliferation with an IC_{50} of 34.1 μM (95% CI 30.1 to 38.5 μM), a small but statistically significant improvement over SL0101. To test the hypothesis that the alkoxyacetone substitution of the acetates found in SL0101 would confer greater biological stability, this new analogue was evaluated in the in vitro biological stability assay described under Task 1c (figure 4). Unexpectedly, it did not show improved biological stability in vitro.

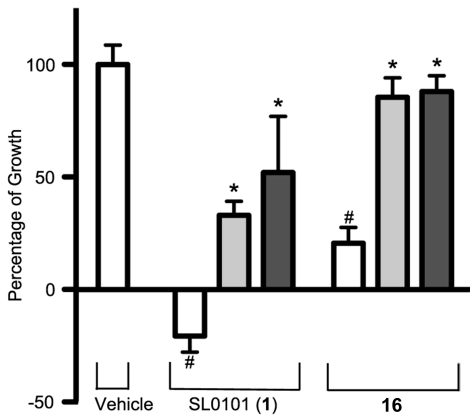


Fig 4. In vitro determination of inhibitor stability. For a detailed description, please see Fig 2 legend. Analogue **16** did not exhibit improved stability compared to SL0101.

Task 3: Synthesis and biological evaluation of conformationally restricted analogues of SL0101. (Timeframe: months 3-12)3a. Synthesis of a conformationally restricted analogue retaining a 3'-carbonyl, or related analogue. (months 3-9)

3b. Synthesis of a conformationally restricted analogue retaining a 4'-carbonyl, or related analogue. (months 3-9)

New data obtained since this task was written suggested that the proposed conformationally restricted analogues were not the ideal analogues to make and that an alternative approach to make the "related analogues" specified in the task would lead to a higher likelihood of successfully identifying a SL0101 analogue with the desired properties. Specifically, in collaboration with the Derewenda laboratory at the University of Virginia we have obtained an X-ray crystal structure that reveals how SL0101 binds to the N-terminal kinase domain of RSK2 (Figure 5). Significantly, this crystal structure shows that SL0101 binds to a type of allosteric site on RSK that is novel and unexpected, making this crystal structure an important contribution to the field of structural biology. A manuscript detailing this work has been accepted for publication in the journal *Biochemistry* (9). Importantly, this new information about the structure of SL0101 bound to RSK makes obsolete the docking studies previously used to propose new analogues, and suggests that some of the originally proposed analogues should not be made while other, related analogues should be prioritized.

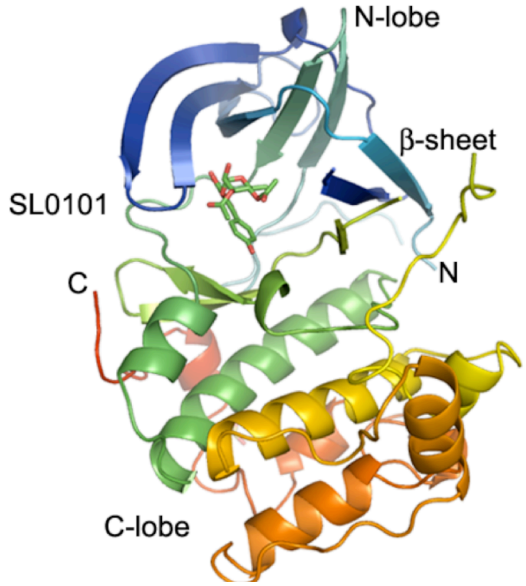
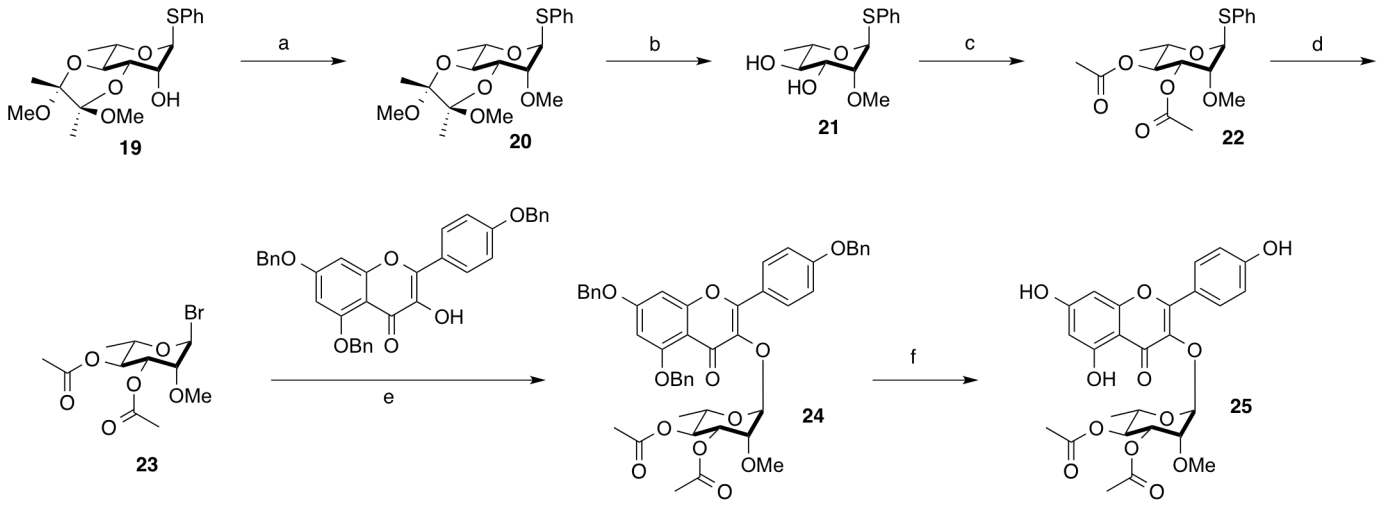


Fig 5. A crystal structure of SL0101 in complex with the NTKD of RSK2. SL0101 binds to a novel allosteric site.

The hypothesis that led to the design of the analogues to be synthesized in Task 3 was that the 3'- and 4'- carbonyl groups of SL0101 accept hydrogen bonds from RSK when SL0101 binds to RSK. The crystal structure of the SL0101-RSK complex reveals that this is not the case. Therefore, the proposed analogues would likely not be more potent SL0101 and to synthesize and test them would be an unproductive use of resources. In contrast, the crystal structure revealed that the 2'-hydroxyl group of SL0101 accepts a hydrogen bond when bound to RSK. This suggested a potentially more fruitful class of analogues that would be predicted to have greater potency than SL0101 in a cell proliferation assay. Since the 2'-hydroxyl group is a hydrogen bond *acceptor* (requiring only the oxygen atom of the hydroxyl group) and not a hydrogen bond *donor* (requiring both the hydrogen and oxygen atoms of the hydroxyl group), one intriguing hypothesis was that the hydrogen of the hydroxyl group could be replaced by an alkyl group as in a methyl ether (**25**, Scheme 5). Such an analogue would be predicted to be as potent as SL0101 in the in vitro kinase assay, but potentially more potent than SL0101 in the cell proliferation assay, as the added lipophilicity conferred by the alkyl substitution could enhance the ability of the analogue to pass through the cell membrane. I was able to synthesize this analogue within the allotted timeframe by making a simple

modification to the existing synthesis of SL0101, whereby instead of alkylating intermediate **19** with benzyl bromide, I instead alkylated it with iodomethane. The rest of the synthesis proceeded analogously to the synthesis of SL0101 (*6*), providing 2'-methyl ether analogue **25**.



Scheme 5. Synthesis of a 2'-methoxy analogue of SL0101. Reagents and conditions: (a) NaH, THF, 0 ºC then CH_3I, reflux, 72%; (b) TFA:H_2O (10:1), CH_2Cl_2, rt, 83%; (c) Ac_2O, Et3N, DMAP, CH_2Cl_2, rt, 88%; (d) Br_2, CH_2Cl_2, 0 ºC, 57%; (e) Ag_2O, 4 Å MS, CH_2Cl_2, rt, 89%; (f) H_2, $Pd(OH)_2$/C, MeOH, EtOAc, rt, 29%.

3b. In vitro evaluation of analogues (months 9-12)

The new analogue **25** was evaluated for its ability to inhibit RSK2 activity in an in vitro kinase assay. Initial experiments indicated that this ether analogue inhibited RSK2 activity with an IC_{50} of 0.488 μM (95% CI 0.178 to 1.34 μM), indicating that it is equipotent to SL0101 and confirming that the 2'-hydroxyl group can be alkylated without interfering with the ability of an analogue to inhibit RSK.

3c. Evaluation of growth inhibition activity of analogues in human cancer MCF-7 and normal human MCF-10A cell lines. (months 9-12)

Analogue **25** was evaluated for its ability to inhibit the growth of the breast cancer cell line MCF7. Preliminary results suggested that the new analogue is approximately 2-fold more potent than SL0101 at inhibiting the growth of MCF7 cells, and that the analogue is, like SL0101, unable to inhibit the growth of the normal breast cell line MCF-10A. These results were further confirmed when this series of analogues was later revisited (see discussion of Task 4e).

Task 4: Design, synthesis, and biological evaluation of potentially more potent SL0101 analogues that append lipophilic groups from the flavone ring system, or related analogues. I will first prioritize analogues based on a computational model. I will then synthesize one or more analogues and evaluate their biological activity. If warranted, I will then synthesize additional related analogues. (Timeframe: months 9-24)

4a. Model potential analogues using ICM-Pro or related software. Potential analogues will be docked into ATP binding site of human RSK using the crystal structure of RSK1 N-terminal kinase domain bound to staurosporine or related crystal structure. (months 9-12)

This subtask was completed in the allotted timeframe using the crystal structure of SL0101 in complex with RSK obtained in collaboration with the Derewenda group. Based on information gleaned from the crystal structure, SL0101 analogues that append lipophilic groups from the flavone ring system as proposed in the project narrative would be expected to be substantially less potent than SL0101. This conclusion is supported by computer-aided docking studies of the proposed analogues into the SL0101 binding site (Figure 6). Analogues were docked using the dock function of the Molecular Operating Environment (MOE) software package published by Chemical Computing Group (CCG) in Montreal, Canada. The program assigns a docking score and RMSD for each proposed analogue. The docking score for SL0101 is -7.96. Compounds that have a lower docking score would be predicted by the program to be more potent inhibitors of RSK than SL0101, whereas compounds with higher docking scores would be expected to be less potent. The RMSD is a measure of how closely the analogue overlaps with SL0101 as positioned in the crystal structure. An RMSD of 1 would indicate perfect overlap, so a lower RMSD indicates confidence by the program that an analogue would bind similarly to SL0101. The originally proposed N-alkylated analogue (**26**, Figure 6) has both a very poor docking score (-6.28) and RMSD (4.12). This is consistent with the qualitative observation that the N-methyl group would cause an unfavorable Van der Waals clash within the binding pocket. Given this information, a set of related analogues (**27**-**29**) were docked and show improved docking scores and RMSD values. Only a representative subset of proposed analogues that were docked are shown.

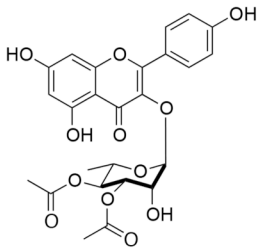
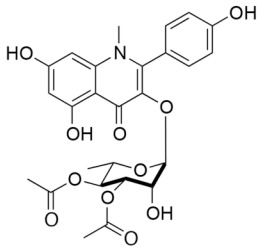
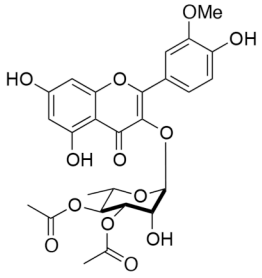
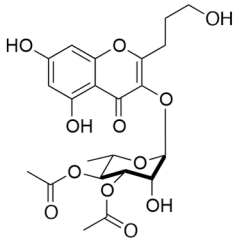
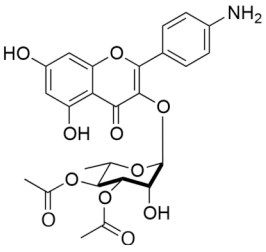
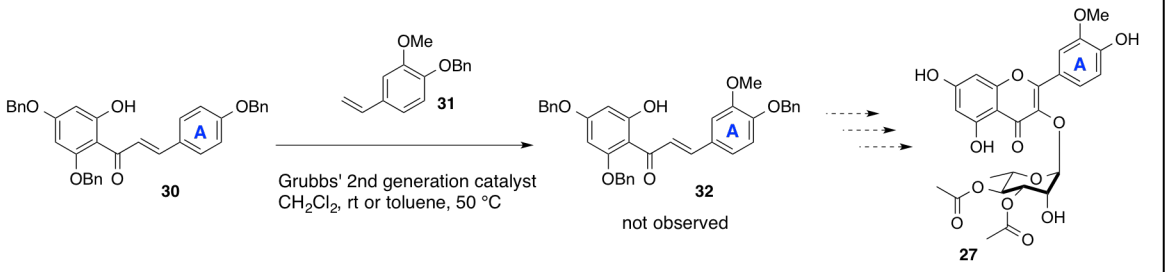
Compound #	Structure	Docking Score	RMSD
1		-7.96	1.63
26		-6.28	4.12
27		-8.07	1.95
28		-7.74	3.32
29		-7.95	1.44

Fig 6. Docking results for proposed analogues obtained using the MOE softwarpackage.

4b. Synthesis of an initial analog or set of analogs prioritized based on modeling results. (months 12-15)

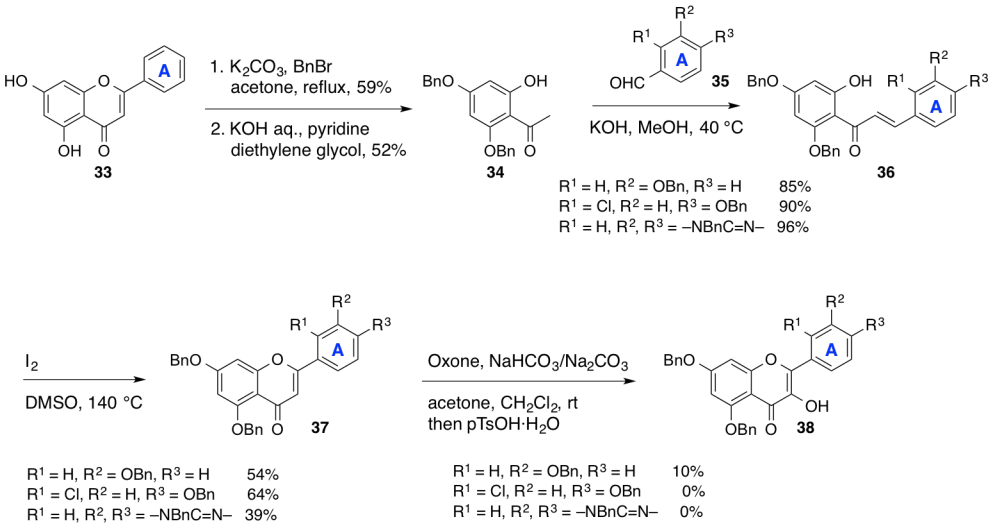
This task was completed within the allotted timeframe. The modeling results, a subset of which are shown in figure 6, suggested that structural changes to the A-ring of SL0101 could lead to analogs with improved binding to the NTKD of RSK2 and by extension improved ability to inhibit RSK activity. Analogue **27**, having the lowest docking score of this subset of analogue candidates, was chosen as the initial synthetic target. Due to the commercial availability of styrene **31** a cross-metathesis strategy starting from known SL0101 intermediate **30** was chosen as an initial approach to A-ring analogues (Scheme 6). Several different

conditions were attempted for the cross metathesis reaction (varying solvent and temperature) however in no case was the desired product **31** observed. Cross metathesis attempts with other alkenes were also unsuccessful (not shown).



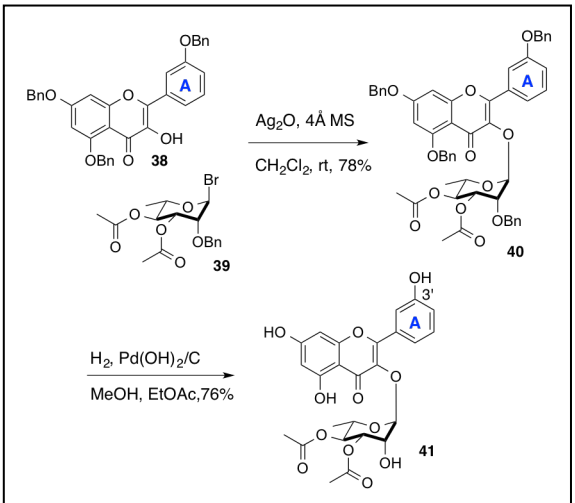
Scheme 6. An initial attempt at synthesizing A-ring analogues of SL0101 utilizing an olefin cross-metathesis strategy

In order to complete an initial A-ring analogue a revised synthetic strategy was employed (Scheme 7). The key step to install a new A-ring moiety was an aldol condensation between ketone **34** and aldehydes **35**. Initial aldehydes used in this reaction were selected based on commercial availability and the docking results for the proposed A-ring analogue (not shown). The initial aldol condensation proceeded smoothly and in high yield for all cases, providing enones **36**, which were then cyclized to provide flavones **37**. Unexpectedly hydroxylation to provide the completed "top piece" intermediates **38** gave none of the desired product for two of the modified flavones and only 10% yield for the third.



Scheme 7. A revised approach to A-ring analogues of SL0101

Despite the poor yield for the previous step I was able to complete the synthesis of an initial A-ring analogue of SL0101 in which the phenolic hydroxyl group of the A-ring is relocated to the 3' position of the A-ring. Glycosylation of intermediate **13** followed by removal of the four benzyl protecting groups provided completed analogue **16** in good yields for both steps (Scheme 8).



Scheme 8. Completion of an A-ring analogue

4c. In vitro evaluation of analog or analogs. (months 15-18)

I completed this task within the allotted timeframe. I evaluated A-ring analogue **41** in an in vitro kinase assay. It inhibited RSK2 kinase activity with an IC_{50} of 43.5 μM. Analogue **41** was 25-fold less potent than SL0101 (IC_{50} = 1.7 μM) in the same assay. This large decrease in potency was surprising since the docking results based on the crystal structure of SL0101 bound to RSK2 suggested that it should be

roughly equipotent, if the ability of an analogue to bind to RSK2 correlates with inhibitory activity. This result suggests a need to reevaluate the use of docking scores for selection of SL0101 analogue targets.

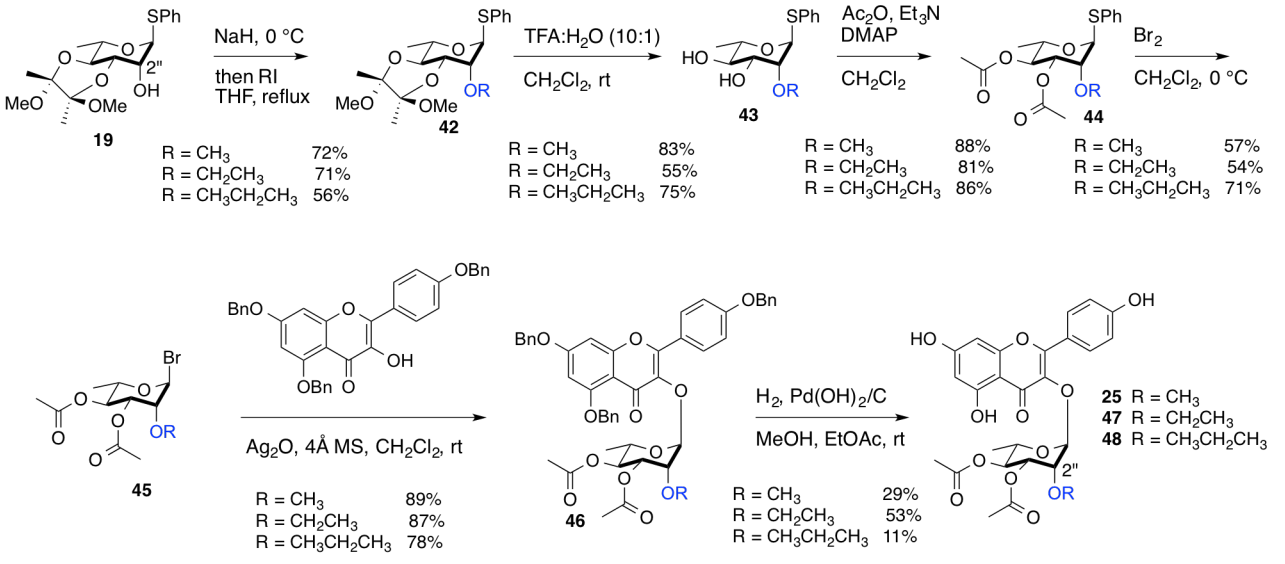
4d. Evaluation of growth inhibition activity of analogs in human cancer MCF-7 and normal human MCF-10A cell lines. (months 15-18)

A preliminary evaluation of the ability of analogue **41** to inhibit the growth of MCF-7 cells indicated that it was substantially less potent than SL0101, showing essentially no effect on growth up to a concentration of 100 μM. As a consequence, I decided not to proceed with the further biological evaluation and instead focus on developing other analogues with the chance to be superior to SL0101 (see subtask 4e).

4e. If warranted, synthesis and biological evaluation of additional related analogs. (months 18-24)

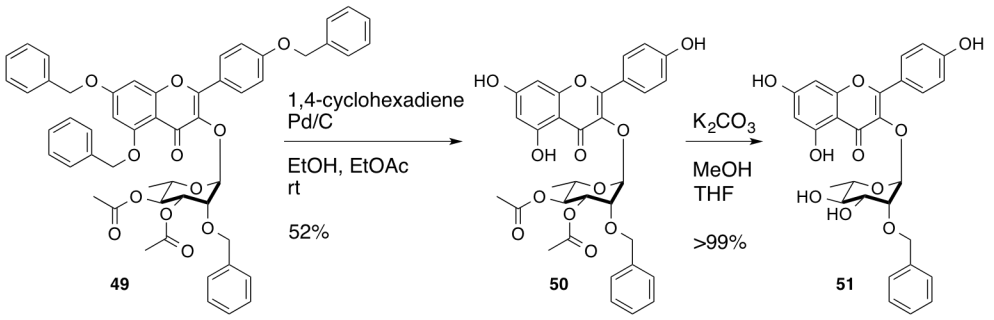
I completed this task within the allotted timeframe. Due to the poor biological activity of analogue **41**, I decided to refocus my efforts on a series of analogues I first explored during Year 1 of the project. I previously demonstrated that the 2" hydroxyl group of the rhamnose portion of SL0101 could be replaced with a methyl ether (analogue **24**, Scheme 4) and that the new analogue is indistinguishable from SL0101 in its ability to inhibit RSK. The rationale behind the design of this analogue was that the crystal structure of SL0101 bound to the NTKD of RSK2 shows that the C2" hydroxyl group of SL0101 accepts a hydrogen bond from a nearby lysine, rather than using its hydrogen to donate a hydrogen bond. My hypothesis was that since the hydrogen of the hydroxyl group was not necessary, alkylation to form an ether would not be detrimental to an analogue's ability to inhibit RSK. Furthermore, an ether at that position would increase the lipophilicity of the analogue overall and potentially increase its ability to enter cells. The outcome of this would be improved potency of the analogue in cell-based assays. Indeed, I disclosed in my last annual report that analogue **25** in initial proliferation assays with MCF-7 cells was roughly twice as potent as SL0101.

Based on these initial results I decided that additional analogues in this series were warranted to continue to try to improve the potency of SL0101 in cell-based assays. Toward this end I re-synthesized analogue **24** and synthesized two new analogues **25** and **26** (Scheme 9), incorporating ethyl and n-propyl ethers, respectively. The synthetic route used to access these analogues is shown in Scheme 4. The key diversification step, namely an alkylation of intermediate **17** with various alkyl halides to provide ethers **18**, proceeded smoothly and in moderate to good yields. The remainder of the synthesis was unchanged from the synthetic route to SL0101, and proceeded without incident to provide completed 2" ether analogues **24**–**26**.



Scheme 9. The synthesis of C2"-ether analogues of SL0101

To add to the collection of C2" ether analogues of SL0101 I synthesized two additional analogues using a different synthetic route. Under transfer hydrogenation conditions I was able to selectively remove the phenolic benzyl protecting groups of known intermediate **49** while leaving the C2" benzyl ether intact, providing C2" ether analogue50 (Scheme 10). The acetate esters of analogue **50** were also hydrolyzed, providing additional diol analogue **51**.



Scheme 10. Synthesis of benzyl ether analogues

All of the new C2" ether analogues were then evaluated for the ability to inhibit both RSK2 kinase activity (Table 1). Diol analogue **51** did not demonstrate any ability to inhibit RSK so it is not included in the table. All of the acetylated analogues were able to inhibit RSK2 kinase activity, however the trend observed was for decreasing potency as the size of the C2" ether substituent increased. However, the initial hypothesis was that due to increased lipophilicity these analogues would be better able to penetrate the cell membrane and would exhibit higher potency in ex vivo (cellular) assays. The ability of all new C2'' ether analogues to inhibit the proliferation of the MCF7 breast cancer cell line was determined (Table 2). A general trend was observed wherein the IC_{50} decreased with increasing lipophilicity of the C2'' ether substituent. Notably, all of the new C2'' ether analogues were more potent than SL0101 in this assay. Since the ether analogues did not exhibit increased potency for inhibition of RSK, these data are consistent with the hypothesis that increased lipophilicity would enhance the ability of the analogues to penetrate the cell membrane.

compound	*RSK2 IC_{50} (μM)*	*p*(**1**)	MCF7 IC_{50} (μM)	*p*(**1**)
1	0.949 ± 0.330		60.2 ± 7.2	
25	0.646 ± 0.265	>0.05	35.5 ± 23.0	>0.05
47	4.008 ± 0.984	<0.01	4.18 ± 1.97	<0.01
48	17.04 ± 13.40	<0.01	9.57 ± 0.59	<0.01
51	36.10 ± 5.926	<0.01	7.21 ± 2.54	<0.0

Table 2. Potency of SL0101 and analogues in in vitro kinase and MCF-7 proliferation assays. IC_{50} is concentration needed for 50% inhibition; n=2 in triplicate; meand and SD, p(**1**) is unpaired Student t-test compared to SL0101 (**1**)

An alternative explanation for the increased MCF7 cell proliferation assay potency of these compounds in light of their modest RSK inhibitory activity is that they are no longer specific for RSK. If true, this could be highly detrimental to their potential as breast cancer drugs due to off-target biological activity. Before moving forward with additional analogues based on this scaffold, we needed to first determine whether RSK specificity was maintained. Initially, determined the ability of the most potent analogues, **47**, **48**, and **49**, to inhibit the proliferation of the normal breast cell line MCF-10A (Figure 7). It has previously been reported that the analogues that are

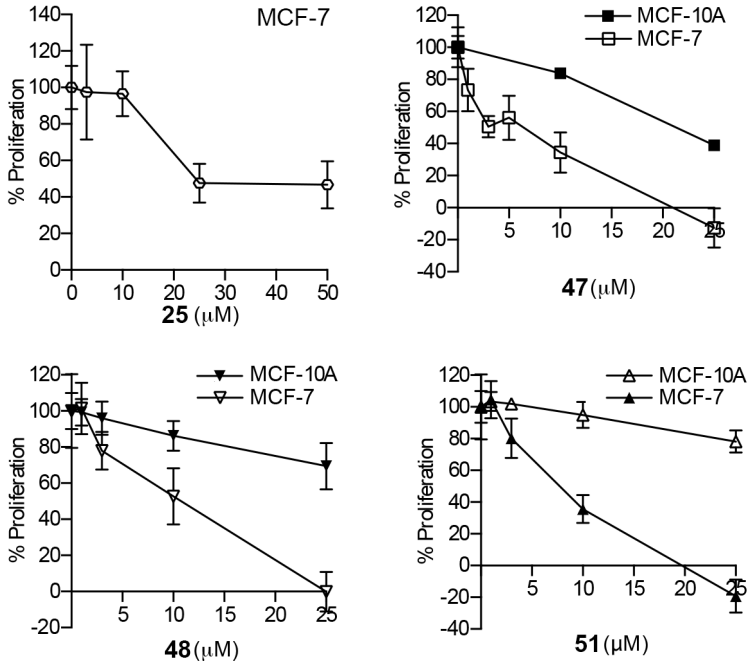
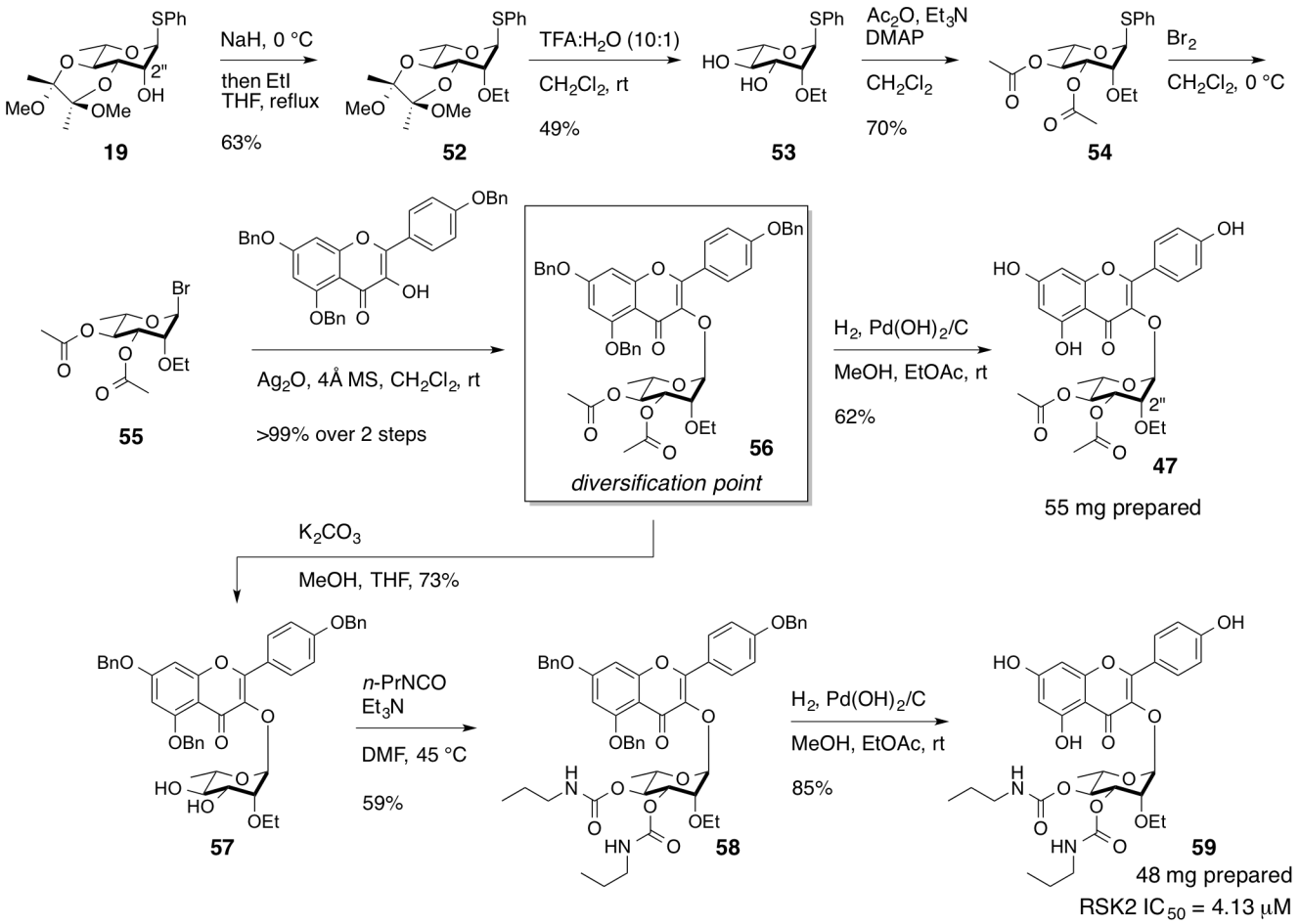


Fig. 7. Analogues **47, 48,** and **51** selectively inhibit MCF-7 over MCF-10A cell proliferation. MCF-7 or MCF-10A cells were treated with vehicle or the indicated concentration of the indicated analogues. % Proliferation shown is normalized to treatment with vehicle.

specific for RSK exhibit a greatly reduced potency to inhibit the growth of non-transformed cell lines (3). The analogues did not inhibit the growth of the MCF-10A cell line at their respective IC_{50}'s for inhibition of MCF7 proliferation, providing evidence that they retained specificity for RSK. As a further measure of RSK specificity, we looked at a downstream marker of RSK inhibition in MCF7 cells. In particular, RSK negatively regulates eEF2 kinase. As a consequence of inhibition of cellular RSK, peEF2 levels increase. Upon treatment of MCF7 cells with SL0101 or all four ether analogues at 100 μM concentration a substantial increase in peEF2 levels were observed by Western blotting (not shown). At lower concentrations, the effect of analogues on peEF2 levels was consistent with their relative ability to inhibit cancer cell proliferation. These data further supported the conclusion that in this set of analogues, specificity for RSK is maintained.

With this information in hand, we decided to select a single analogue to serve as a platform for the development of a new RSK analogue that combines structural features that improve potency with structural features that improve biological stability. This analogue would then be suitable for in vivo work if potency and selectivity for RSK were maintained. We determined that analogue **47** exhibited the best combination of RSK inhibitory potency and cell proliferation potency, and therefore endeavored to synthesize an analogue incorporating both an ethyl ether at the 2' position and *n*-propyl carbamates at the 3' and 4' positions, which we had previously determined to confer the ideal combination of potency and biological stability. In order to provide enough material for extensive in vitro and later in vivo studies, we targeted the synthesis of 50 mg each of both the new carbamate analogue **59** and analogue **47** (to be used as a comparison). To achieve the most efficient synthesis of each analogue we devised a synthetic route that could produce both analogues (Scheme 11). Starting from known rhamnose derivative **19**, alkylation with iodoethane followed by deprotection under acidic conditions gave diol **53**. Acetylation followed by treatment of the resulting diacetate with bromine provide glycosyl bromide **55**, which was coupled to the protected flavonol. The resulting advanced intermediate **56** was used to prepare analogue **47**. In addition, new carbamate analogue **59** could be prepared from intermediate **13**. Hydrolysis of the 3' and 4' acetates was followed by formation of the carbamate **58**. The



Scheme 11. Resynthesis of analogue **3** and synthesis of a new analogue **16** combining a 2''-ether modification with 3'' and4'' carbamate modifications, previously shown to confer improved biological stability.

analogue synthesis was completed after hydrogenolysis of the benzyl protecting groups to provide **59**. Analogue **59** was tested in the in vitro kinase assay for its ability to inhibit RSK2 activity, and was determined to be equipotent to the corresponding diacetyl analogue **47**. Additionally, the ability of new analogue **59** to inhibit the proliferation of both MCF-7 and MCF-10A cells was evaluated. The analogue was approximately 15-fold more potent than SL0101 at inhibiting the growth of the cancer cell line. Additionally, it did not inhibit the proliferation of the normal cell line at its IC_{50} for MCF-7 proliferation, providing evidence for RSK (Figure 8).

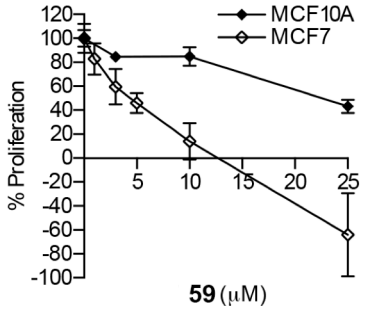


Fig. 8. Analogue **59** selectively inhibits MCF-7 over MCF-10A cell proliferation. MCF-7 or MCF-10A cells were treated with vehicle or the indicated concentration of analogue **59**. % Proliferation shown is normalized to treatment with vehicle.

Task 5: In collaboration with Michelle Rudek-Renaut, evaluate the biological half-life of 1-3 analogs in CB17 SCID mice for both subcutaneous and intraperitoneal routes. (Timeframe: months 1-6 and 21-27)

Task 6: Evaluation of up to 3 analogs in our breastoid model. This will require the use of 15 human tissue samples, which we will collect under an approved IRB protocol that protects patient identity. (Timeframe: months 1-6 and 28-36)

In order for tasks 5 and 6 to be scientifically justified, an analogue with suitable potency, specificity for RSK, and in vitro biological half-life must first be identified. We decided to move forward with further in vitro and ex vivo evaluation of analogues **47** and **59** in order to gain more information about in vitro biological half-life and specificity for RSK to confirm that they were suitable for in vivo evaluation and evaluation in the breastoid model. These analogues were chosen based on their combination of potency for inhibition of RSK and for inhibition of MCF-7 cell proliferation, and for the initial promising results for RSK selectivity.

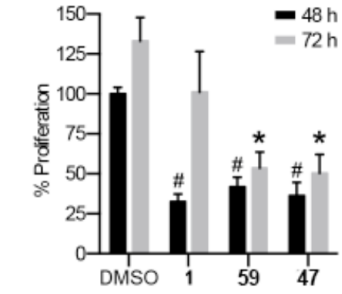


Figure 9. Stability of compounds **59** and **47** in cell-based assays. MCF-7 cells were treated for 48 and 72 hours with 100 μM SL0101 (**1**), 10 μM (**59**) and 25 μM (**47**). . (n=2 in triplicate; mean, S.D.; # $p<0.01$ compared to MCF-7 control at 48 h.* $p<0.01$ compared to MCF-7 control at 72 h)

Initially we evaluated the ex vivo biological half life of **47** and **59**. To compare their stability to that of SL0101 (**1**) of in cell-based assays we measured their ability to inhibit proliferation over a 72 h time frame. As expected, SL0101 inhibited proliferation for 48h but then the cells started to proliferate (Figure 9). In contrast, analogues **47** and **59** inhibited proliferation throughout the assay. This result was expected for analogue **59** (which was predicted to be more stable due to the presence of the carbamate groups), but not for analogue **47**.

The selectivity of analogs for RSK in cells can be further determined by observing phosphorylation levels of signaling components that are downstream of RSK. We tested analogue **47** at the cytostatic concentration of 25 μM and **59** at the cytostatic concentration of 10 μM and the cytotoxic concentration of 25 μM. We have previously determined that RSK regulates the levels of the cell cycle regulator, cyclin D1. SL0101 and analogues **47** and **59** decreased cyclin D1 levels (Figure 10). To evaluate other RSK downstream effectors we determined whether the phosphorylation of eukaryotic elongation factor 2 (eEF2) was altered by the analogues. RSK inhibits the activity of eEF2 kinase and thus inhibition of RSK activates eEF2 kinase, which results in the phosphorylation of its substrate eEF2 (p-eEF2). As previously observed SL0101 at its IC_{50} as a cytostatic agent increased p-eEF2 levels but 25 mM of **59,** which is a cytotoxic dose**,** only induced a modest increase (Figure 4).

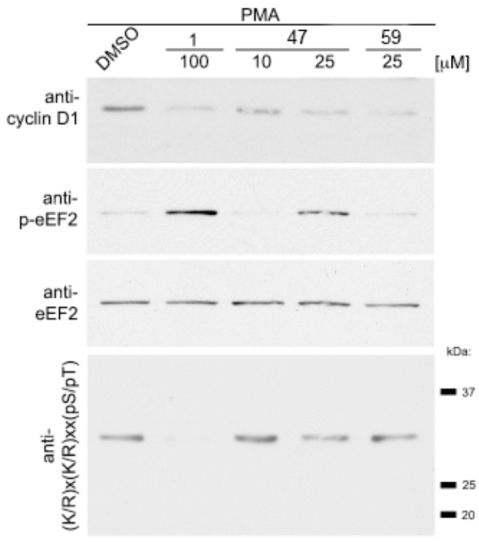


Figure 10. Evaluation of specificity of analogs **47** and **59** compared to SL0101 (**1**). MCF-7 cells were pre-treated with inhibitors for 16 hours,and then treated with PMA. Equal amounts of total protein lysate were immunoblotted.

Concentrations of **59** and **47** that resulted in inhibition of proliferation had no effect of p-eEF2 levels. To further evaluate specificity we used an antibody against a phosphorylation motif, which is recognized by a subset of the AGC family kinases, which includes RSK. SL0101 inhibited the phosphorylation of a band at ~ 27 kDa but **47** and **59** had no effect. Taken together, these results suggest that analogues **47** and **59** inhibit MCF-7 proliferation via down regulation of cyclin D1 but that this decrease is due to a RSK-independent pathway.

To shed further light on the selectivity of SL0101 and analogue **59** we submitted them to a kinase selectivity screen (SelectScreen from Life Technologies) and also evaluated their selectivity using phosphoantibody arrays acquired from Full Moon Biosystems. This served two purposes: (1) to determine whether **59** was inhibiting some other kinase which would be a potential target for breast cancer treatment, given that treatment of MCF-7 cells with **59** resulted in a downregulation of cyclin D1 levels; and (2) to gain further insight into the selectivity of SL0101 for RSK, since a complete evaluation of its kinase selectivity (for example against a panel of 414 different kinases in the SelectScreen panel) had never been obtained. The SelectScreen results (obtained using 5 μM inhibitor concentration at the Km app for each kinase) indicated that while SL0101 is indeed selective for RSK isoforms, it is selective within the RSK family (no observed inhibition of RSK3 at the tested concentration). In addition, several other kinases that SL0101 inhibits as well as it inhibits RSK2 were identified for follow-up experiments, which are ongoing. The lack of selectivity of analogue **59** for RSK was confirmed; the initial screening revealed that it inhibits several kinases, including DCAMKL2 and MUSK to a greater extent than it inhibits RSK. Data from the phostphoantibody array experiments have been collected a network analysis of the data is in progress. Taken together, the data on the selectivity of the new analogues suggest that they are not selective for RSK. Therefore, tasks 5 and 6 were not scientifically justified.

Training plan:

Task 1: Audit "Advanced Topics in Cancer" class. (Timeframe: 1-4 months)

I audited the "Advanced Topics in Cancer" class in year 1.

Task 2: Audit "Cell Imaging" class. (Timeframe: 4-8 months)

Rather than Audit the "Cell Imaging" class I received personal advice and instruction on cell imaging techniques from the Director of the Advanced Microscopy Facility at the University of Virginia, Ian Macara.

Task 3: Regularly attend cancer and chemistry seminars. (Timeframe: 1-36 months)

I regularly attended cancer seminars offered by the Cancer Center at the University of Virginia and chemistry seminars offered by the University of Virginia Department of Chemistry during the project.

Task 4: Attend international meeting held in the US to present and discuss work. (Timeframe: 1-12 months)

I attended the American Society for Cell Biology National Meeting, the Cancer Biology Training Consortium annual Chair & Director's Retreat, and the Mid-Atlantic Regional Meeting of the American Chemical Society.

Task 5: Attend international meeting held in the US to present and discuss work. (Timeframe: 13-24 months)

Since I attended three meetings during Year 1 of the project rather than the planned one, I chose not to attend a meeting during Year 2.

Task 6: Attend international meeting held in the US and BCRP Era of Hope meeting to present and discuss work. (Timeframe: 25-36 months)

The BCRP Era of Hope meeting was not held.

Personnel Receiving Pay From the Research Effort: Michael Hilinski

4. Key Research Accomplishments

- The discovery of analogues of the RSK inhibitor SL0101 that are more biologically stable than the parent compound in vitro yet retain specificity for RSK. These analogues are candidates for in vivo evaluation.
- The solution of an X-Ray crystal structure of SL0101 in complex with the NTKD of RSK2. This structure will be an invaluable tool for the design of new SL0101 analogues.
- The discovery of an analogue of the RSK inhibitor SL0101 that is approximately 15 times more potent than SL0101 at inhibiting the growth of breast cancer cells. Despite its lack of selectivity for RSK, the regulation of cyclin D1 levels by this analogue suggests that further experiments into its mode of action are warranted.

5. Conclusion

SL0101 is a promising lead compound for medicinal chemistry efforts to develop a breast cancer drug that works by targeting RSK. However it suffers from poor biological stability and potency, making it unsuitable for use as a drug. The discovery of analogues of SL0101 that are more biologically stable and that are more potent in cell-based assays as described in this report is thus highly significant as they overcome these deficiencies and therefore could ultimately find use as breast cancer drugs. Future plans include the continued evaluation of the selectivity of SL0101 and the analogues that have been synthesized for kinase selectivity; thus identifying kinases of concern for off-target activity and other target kinases for cancer treatment. This information will be highly useful for the design of a breast cancer drug that works by inhibiting RSK selectively.

6. Publications, Abstracts, and Presentations

- Publication: “Analogues of the RSK Inhibitor SL0101: Optimization of In Vitro Biological Stability” **M. K. Hilinski**, R. M. Mrozowski, D. E. Clark, D. A. Lannigan, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3244–3247. PubMed PMID: 22464132
- Publication: “Insights into the Inhibition of p90 Ribosomal S6 Kinase (RSK) by the Flavonol Glycoside SL0101 from the 1.5 Å Crystal Sturcure of the N-Terminal Domain of RSK2 with Bound Inhibitor” D. Utepbergenov, U. Derewenda, N. Oleknovich, G. Szukalska, B. Banerjee, **M. K. Hilinski**, D. A. Lannigan, P. T. Stukenberg, Z. S. Derewenda, *Biochemistry* **2012**, *51*, 6499–6510. PubMed PMID: 22846040
- Publication: Mrozowski, R. M.; Vemula, R.; Wu, B.; Zhang, Q.; Schroeder, B. R.; **Hilinski, M. K.**; Clark, D. E.; Hecht, S. M.; O’Doherty, G. A.; Lannigan, D. A. “Improving the Affinity of SL0101 for RSK Using Structure-Based Design” *ACS Med. Chem. Lett.* **2013**, *4*, 175–179. PubMed PMID: 23519677
- Presentation: “Selective Inhibitors of RSK as Anticancer Drug Leads: A Medicinal Chemistry Story” *University of Virginia Cancer Center Seminar Series* **November 14, 2014**, Charlottesville, VA.

7. Inventions, Patents, and Licenses

Nothing to report.

8. Reportable Outcomes

- Employment received based on training supported by this award: The P.I., Michael Hilinski was hired as an Assistant Professor in the Department of Chemistry at the University of Virginia.

9. Other Achievements

Nothing to report.

10. References

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Appendices

none